Use of reflex indicators for measuring vitality and mortality of *Chionoecetes opilio* (snow crab) in captivity.

Running title: Measuring vitality in snow crabs

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Keywords: Reflex indicator assessment, snow crab, air exposure

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Abstract

The snow crab (*Chionoecetes opilio*) is one of the most important commercial crabs in the world, it is heavily exploited in Atlantic Canada, Alaska, the Sea of Japan and the Barents Sea. Catches in the Barents sea north of Norway have increased dramatically in the last decade. Most of the world's catch is processed, frozen and exported overseas. However, recently there has been considerable interest in exporting live snow crab, particularly in Norway. The stress of capture and live transport can result in significant mortalities. In order to establish a live export industry for snow crab, the welfare of the animal must be monitored throughout all the steps of the live transport process. In the current experiment the reactions of snow crabs exposed to increasing periods of air exposure were measured in terms of appropriate reflex indicators, incidence of mortality, blood lactate levels and blood protein and hemocyinin. The aim was to test the suitability of reflex indicators to reflect vitality (stress) and not just to predict mortality. This would be compared with traditional blood biochemistry techniques for measuring crustacean stress. The study demonstrated that the reflex index score is suitable to assess the vitality of snow crab. Longer air exposure periods render higher mortality rates and less vital individuals. The authors believe using vitality reflex indicators would be a suitable way of measuring crab

welfare throughout the live holding and transport process. Further research is required to refine the technique and to test reflex indices against other crustacean species.

Keywords

Reflex indicators, vitality, mortality, *Chionoecetes opilio,* snow crab

Introduction

The snow crab (*Chionoecetes opilio*) is one of the most important commercial crabs in the world, it is heavily exploited in Atlantic Canada, Alaska and the Sea of Japan (Bailey and Elner, 1989). The snow crab is an invasive species in the Barents Sea and was first observed in 1996 (Kuzmin *et al*., 1999). Furthermore, Alsvåg *et al*., (2009) described self-sustainable populations of snow crab in the Barents Sea in 2009 and subsequently the species has developed into a significant fishing resource in the Barents Sea. In 2014 landings in Norway were 4000t and in 2015 exceeded 9000t and future catches are estimated to be significantly higher (Siikavuopio *et al.*, 2017).

Currently most of the Norwegian snow crab catch (and most of the worldwide catch) is processed and sold as frozen product but there is a strong demand and a growing interest in Norway in supplying live snow crab to markets in Asia (Lorentzen *et al.*, 2016; 2018). This is partially a result of the success of live king crab exports from Norway which have increased exponentially in the past decade (Sundet, 2014). As it has been shown for king crab and a range of other crustacean species the stress of capture and live transport can result in significant mortalities if the animal is not cared for correctly throughout the entire value chain. Including when the animal enters the trap, is transported back to shore, is held in live storage facilities, is transported to 'live hubs' for storage and finally during transport to market and live storage and display in the market. Stress is cumulative throughout this process (Jennings *et al*., 2016) and an industry friendly, accurate indicator of vitality (a proxy for stress) and mortality that could be sued throughout the live transport would be a very valuable tool in the development of the live trade for snow crab from Norway and other countries.

RAMP models use reflex actions to score vitality impairment of marine species to predict fisheries bycatch mortality. The RAMP model is constructed using the relationship between vitality impairment and mortality. This technique has been used extensively to predict discard mortality in fish and crustaceans exposed to fishery stressors such as air exposure and changes in temperature and/or depth. An assessment of discard mortality of two Alaskan crab species, the Tanner crab (*Chionoecetes bairdi*) and snow crab (*Chionoecetes opilio*), based on reflex impairment was made by Stoner *et. al. (*2008). In this study six reflex indicators were identified as being useful for assessing stress. The advantages of using RAMP models include their relative low cost, they are simple to use, do not require extensive training or equipment and they are less invasive than sampling blood or taking biopsies from the animal. It is also possible to generate a large sampling number relatively quickly. Although the RAMP model has been extensively used to monitor and predict mortality after fishing it has not been used as a tool for measuring the vitality of crustacean held in captivity (either for fattening post capture or in crab rearing facilities). In the current study the authors investigated whether reflex impairment would be a feasible method of measuring and scoring stress (or vitality) as well as survival in captive snow crabs, based on the reflexes identified by Stoner *et. al.* (2008). Snow crabs were exposed to increasing periods of air exposure and their reflex indicators, incidence of mortality and blood lactate, blood protein and hemocyanin were measured and compared as possible stress indicators.

Materials and Methods

Reflex index assessment

The reflex indicators identified in Stoner (2012) were used in this experiment. These consist of a suite of six reflex actions which are known reflex action mortality predictors (see in Stoner *et* *al.*, 2008; 2009). The combination of all six reflexes (impaired reflex=0, reflex presence = 1) render a reflex index per individual (all six reflexes impaired = 0 while all six reflexes present = 6). In the current experiment 210 male snow crabs (*C. opilio)* were caught by the vessel North-eastern, with snow crab pots in the NEAFC area (74.58 latitude and 38.49 longitude). The crabs were transported live to the Nofima Aquaculture Research Station in Tromsø, Norway (~70°N), and immediately placed in one 6 m^3 tanks supplied with running seawater (~ 3.0 °C, salinity ~ 32). The holding tank were supplied with ambient seawater at ~4°C. The crabs were tagged with T-bar anchor tags (Hallprint Ltd., Victor Harbour, SA, Australia) and sorted in seven groups of 30 individuals. On the first day of experiment, all the crabs were removed from the water and exposed to air at ambient room temperatures between 8 and 10°C. Thereafter, every four hours, one group of crabs was assessed for reflex impairment (i.e. after 0, 4, 8, 12, 16, 20 and 24h of air exposure). Immediately, after being assessed they were returned to the holding tank. After re-immersion in seawater the crabs were assessed again after 48h and after seven days, the proportion of crabs which showed reflex impairment was determined together with the mortality probability (alive crabs/dead crabs x 100).

Blood sampling and testing

Blood samples were collected from individual crabs using a 2ml syringe from the base of the second walking leg. Blood samples were taken from the individuals in groups exposed to air 0h, 12h and 24h. Immediately after collection of the sample the blood lactate was measured using a Freestyle Lite™ blood lactate meter (Abbott, Illinois). Blood samples were also collected, immediately chilled on ice and then frozen at -80°C for later analysis of blood protein and hemocyanin levels. The methodology described in Johnsen *et al.* (1984) were used for the blood analysis.

Statistical analysis

To evaluate the correlation between air exposure, reflex index, blood lactate results and crab mortality, a rank Spearman correlation was run for each variable. Moreover, two groups of crabs were identified: those which died one week after the air exposure period and those which remained alive. A Mann-Whitney *U*-test was performed to determine differences between the mean air exposure and the mean reflex index of both groups of crabs (dead and alive). The power of the reflex index as an indicator of fish vitality was explored using the assumption that longer air exposure periods would reduce fish vitality. A Spearman rank correlation test comparing air exposure time with mean reflex index score was performed. Based on the previous use of lactate as a stress indicator in crustaceans (Lehtonen and Burnett, 2016; Powell *et al*., 2017 and references therein), this was used to corroborate the use of reflex index as an indicator of vitality, assuming that higher levels of lactate will correspond to individuals with lower reflex index (low vitality). The crabs were divided in groups using reflex index as a grouping variable: group 1 (reflex index = 0), group 2 (from 1 to 2), group 3 (3 to 4) and group 5 (5 to 6). Then, a Kruskal-Wallis test was used in seek of differences between mean lactate values obtained for each group of crabs. Possible differences between total blood protein and total hemocyanin levels in crabs after 0hrs (initial), 12 and 24hrs air exposure were compared using a one way ANOVA (NCSS Software, Inc. USA) with distribution of data compared to normal distribution using Shapiro–Wilk test, and homogeneity checked using Levene’s test.

Results

There was no incidence of mortality in snow crab until they were exposed to air for at least 4h with mortality then increasing with increasing exposure ($S = 7.8$, $P = 0.01$; values for instant mortality) and peak mortality values occurred after the maximum 24h of air exposure (Fig.1). A total of 14 crabs died during the exposure period giving a ‘post treatment’ mortality of 6.7%. After 48h of immersion (post treatment) there was a total mortality of 11% and after one week of immersion (post treatment) the total mortality was 15% (Fig.1). Crabs which died after one week were subjected to significantly (W = 771.5; $P < 0.001$) longer periods of air exposure (17.4 ± 5.8h) than crabs which remained alive (10.8 ± 7.7h).

The crabs that died after a week of re-immersion, showed significantly (W = 2144; $P = 0.003$) lower mean values for the reflex index (2.3 ± 1.2) immediately after being exposed to air than crabs which remained alive (3.5 ± 1.6). However, despite the observed positive trend the correlation between mean index values and mortality was not significant (S = 90.58, $P = 0.14$). Individuals that retained more than 4 reflexes did not have any mortality post treatment.

In terms of individuals reflexes, after 48h recovery from the air exposure stress, the alignment, retraction, claw and ventral flap reflexes showed relatively low values (<20%) with values peaking (up to 50%) for crabs which were exposed to air for 24h (Fig.2). In contrast, mortality rates increased for the 'mouth' and the 'eye' reflex impairment after 8h of air exposure, and reached 100% after 16h of air exposure in the 'eye' reflex (Fig.2). Although all the reflexes showed an increasing mortality trend as air exposure increased, the ventral flap reflex was the only which showed a steady increase in mortality over time (r^2=0.88, p-value < 0.05; Fig.2). In terms of the incidence for individual reflexes, a sudden increase for retraction and alignment of legs was observed in crabs right after 4h air exposure with values of 90 – 100% attained after 24h (Fig.2). A similar trend was shown by the claw reflex but the incidence curve over time was smoother. Overall, the mortality values for 'eye' and especially for 'mouth' reflex were much lower compared to the other reflexes, which showed high mortality values from the early stages of air exposure (i.e. retraction, alignment and claw). The ventral flap reflex showed the best correlation between incidence and mortality (r^2=0.62, $P = 0.13$) compared to all other reflexes (range=0.37-0.56) although none of the correlations was significant ($P = 0.13$; Fig.2).

The reflex impairment was a gradual process with limb retraction being the first reflex to disappear followed by limb alignment, claws movement, ventral flap reaction, mouth retraction and eye movement in this order (Fig. 3). Overall, limb retraction was shown by 78.6% of the individuals throughout the trial with an associated mortality of 8.5%. However, eye reflex impairment was shown by the lowest proportion of individuals (10.5%) but mortality peaked at 63.6%. The other reflexes had intermediate values and are shown in Table 1.

Lactate values (mean values ± SD in mmol/L) were measured in the haemolymph of crabs subjected to 0h (0.1 undetectable), 12h (3.5 ± 0.9) and 24h (8.9 ± 2.9) of air exposure. Air exposure resulted in a strong correlation with the mean reflex index (S = 2504000; $P < 0.001$; Fig.4) when the latter was used as a proxy for crab vitality (low reflex index = low vitality). The latter was also consistent with the high correlation (S = 222030, $P < 0.001$) between mean reflex index and lactate (Fig.5). Differences in lactate values between groups of crabs based on reflex index as a grouping variable were found (K = 62.272, df = 3, $P < 0.001$). The highest mean values of lactate were shown by crabs with a reflex index of 0 whereas the lowest lactate values were shown by the crabs which showed mean index values between 5 and 6 (strong vitality score). Crabs with reflex index values between 1 and 4 did show intermediate lactate values and no differences were detected between them (Fig.5).

There were no significant differences in total blood protein (ANOVA: $F_{2, 58} = 0.31$, $P = 0.73$) or total hemocyanin levels (ANOVA: $F_{2, 58} = 0.54$, $P = 0.58$) between crabs exposed to exposed to air for 0h (initial), 12h and 24h.

Discussion

The current experiment tests whether reflex impairment can be used to assess the welfare of a given crustacean throughout the live holding and transport process. This would be considerably simpler, cheaper and a more industry friendly method compared to more traditional analyses of blood biochemistry. The strong correlation between lactate (as a proxy for stress indicator) and the Reflex Index Score (RIS) in the present study demonstrates for the first time that using a RIS is suitable to assess the vitality of captive snow crab. In provides not only information on expected mortality but gives a comparable 'vitality index' based on the RIS that can be used to measure the 'health and welfare' of the animals at any stage of the process. In the current study longer air exposure periods resulted in higher mortality rates and less vital individuals. The study also shows the relationship between air exposure, mortality and each of the 'reflex indices' (6 in total) and how the specific response to the individual 'reflex indices' can be used as stress indicators. The number of 'reflex indices' affected (see Fig. 3) gives an indication of the severity of the stress in addition to the likelihood that mortality will occur.

The relationship between reflex impairment and delayed mortality due to air exposure has been previously studied in a number of crustaceans, although it has been limited to fisheries purposes (Davis, 2007; Davis and Ottmar, 2006; Stoner, 2012, Van Tamelen, 2005; Stoner *et al*., 2008; Chilton *et al*., 2011; Hammond *et al*., 2013; Rose *et al*., 2013). The current study shows that delayed mortality as a result of stress inducing treatments (in this case prolonged air exposure) can increase by two-fold after 48h (post stress and re-immersed in seawater) and nearly three-fold after one week (post stress and re-immersed in seawater). After 4h of constant air exposure mortality occurred in crabs after one week of re-immersion. In this regard, Stoner (2009) found delayed mortality rates around 85% in snow crabs one week after being exposed to air freezing conditions. Paul *et al*., (1994), showed that the stress of 90 days of starvation caused 100% mortality in tanner crabs 140 days after they were offered unlimited feed, meaning that the resilience point was surpassed at some point within the first 90 days of starvation. The latter shows that even if the crabs can tolerate and survive a given stress, the physiological damage might be irreversible and, eventually it may cause death. This is very important for live storage since the fact that the crabs arrive alive at a live holding facility does not guarantee that they will remain alive while being stored, or during the subsequent transport to the final buyer.

The reflex impairment index is obviously a useful predictor for crab vitality. Crustacean condition or 'vitality' has traditionally been measured using analysis of haemolymph chemistry (e.g., lactate, glucose, glycogen levels, blood protein and hemocyanin) (Crear and Forteath, 2001; Engel *et al*., 1993; Harris and Andrews, 2005; Ridgway *et al*., 2006). In the current study there were no significant differences between total blood protein and total blood hemocyanin levels in crabs not exposed to air (0hrs, initial sample) or those exposed to air for 12hrs and 24hrs. This indicates that for air exposure stress over 24hrs these techniques are not good indicators of stress. Based on the correlation between lactate levels and reflex impairment shown in this study, the authors believe that a reflex impairment index using RIS can be used as a reliable vitality indicator for snow crabs throughout the live holding value chain.

In terms of specific reflexes, the crabs showed a marked impairment and incidence pattern which correlated to air exposure time. The loss of reflexes seemed to be related in pairs so the incidence of eye/mouth retraction impairment appeared in extreme stress situation compared to the claws/ventral flap (medium stress situation) and limb's retraction/alignment reflexes in the least stressful situation. The authors suggest that the reflex indices 'retraction' and 'alignment of limbs', the 'retraction of chela' and 'ventral flap', followed by 'mouth' and 'eye' movement, in this order, could be considered as gradually decreasing vitality indicators. The same trend of reflex impairment over time was described (in relation to mortality) for a similar species, the tanner crab (*Chinoecetes bairdi*) (Yochum *et al*., 2015). Raby *et al*., (2012) also showed an equivalent pattern of fish reflex impairment over time with eye-related movement as the last reflex to be impaired. This reflex indicator in fish is now used and recommended by Noble *et al*. (2018) as one of the available tools for assessing fish welfare and the use of reflex impairment index are likely to be useful for a range of stressors, in a wide range of species. However, specific research to calibrate the sort of stress and reflex impairment must be conducted for each species.

The authors believe it would be possible to further develop a numeric scale, based on reflex index scores, to assess vitality of the different crab species. It is likely that a different set of reflex indicators will be required, or will have different value (impact), for each species. In addition, the reflex action score model for snow crab should be further refined to develop a more detailed measurements describing a wider spectrum of vitality. For instance, breaking down each reflex response into sub responses or reducing the number of analysed reflexes to those that best reflect the vitality of the animal.

Overall, more research is suggested in order to implement better management strategies on snow crab industry throughout the value chain. Although further specific research is required the authors believe the technique outlined in this study will be a useful starting point for developing such a system based on reflex indicators for snow crab (*C. opilio*).

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Figures and Table captions

Figure 1. Bar plot depicting the relationship between the air exposure period and mortality of snow crabs (*C. opilio*) after exposure to air. *Light grey*: 'Post treatment' mortality; *Dark grey*: mortality after 48h immersion post treatment; *Black*: mortality after one week immersion post treatment (*n* per time exposure = 30 individuals).

Figure 2. Bar plot depicting the incidence of non-responsive crabs (black dots) and the relative mortality (grey bars) for each of the inspected reflexes in the 24h air exposure treatment.

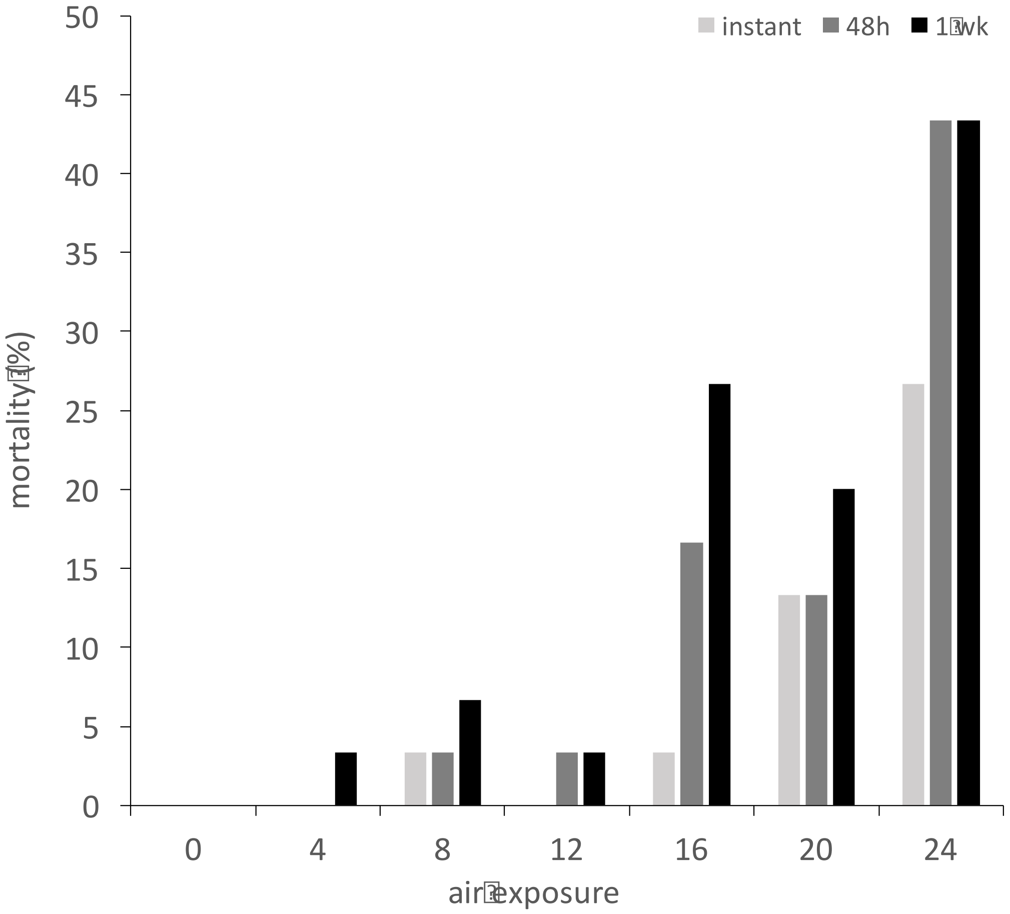
Figure 3. Pie charts depicting the contribution (the proportion of each reflex) to each of the reflex impairment index scores (shown in the centre of each pie chart); *n* = number of individuals showing a given reflex index after the air exposure trial.

Figure 4. Scatter plot (±SE) showing the mean reflex index of snow crabs (*Chionoecetes opilio*), as an indicator of vitality, versus the time exposed to air as a stressor.

Figure 5. Lactate values measured on snow crabs (*Chionoecetes opilio*) after being exposed to air as a function of the individual reflex index.

Figure 6. Total blood protein and Hemocyanin levels in crabs prior to the experiment (0h) and exposed to 12h and 24h air exposure.

Table 1. Proportion of individuals showing reflex impairment together with the relative probability of instant mortality when a reflex is impaired.


instant
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1 wk
mortality (%)
air exposure
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4
8
12
16
20
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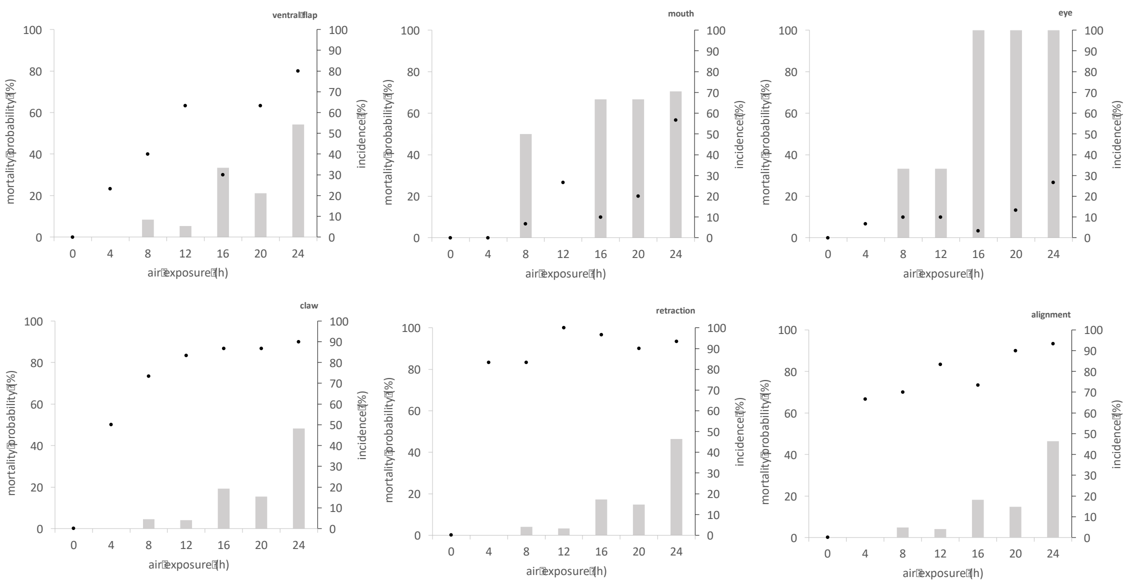
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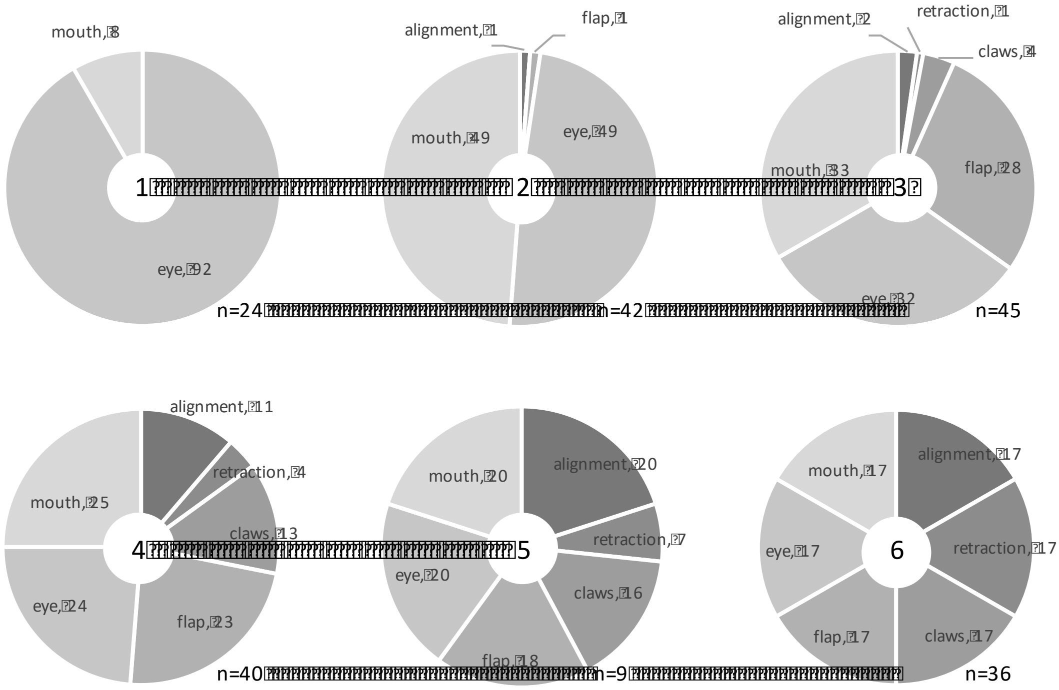
mouth
eye
claw
retraction
alignment
mortality probability (%)
incidence (%)
air exposure (h)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alignment, 1
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eye, 49
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alignment, 2
retraction, 1
claws, 4
flap, 28
mouth, 33
eye, 32
3
n=45
alignment, 11
retraction, 4
claws, 13
flap, 23
eye, 24
mouth, 25
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n=40
alignment, 20
retraction, 7
claws, 16
flap, 18
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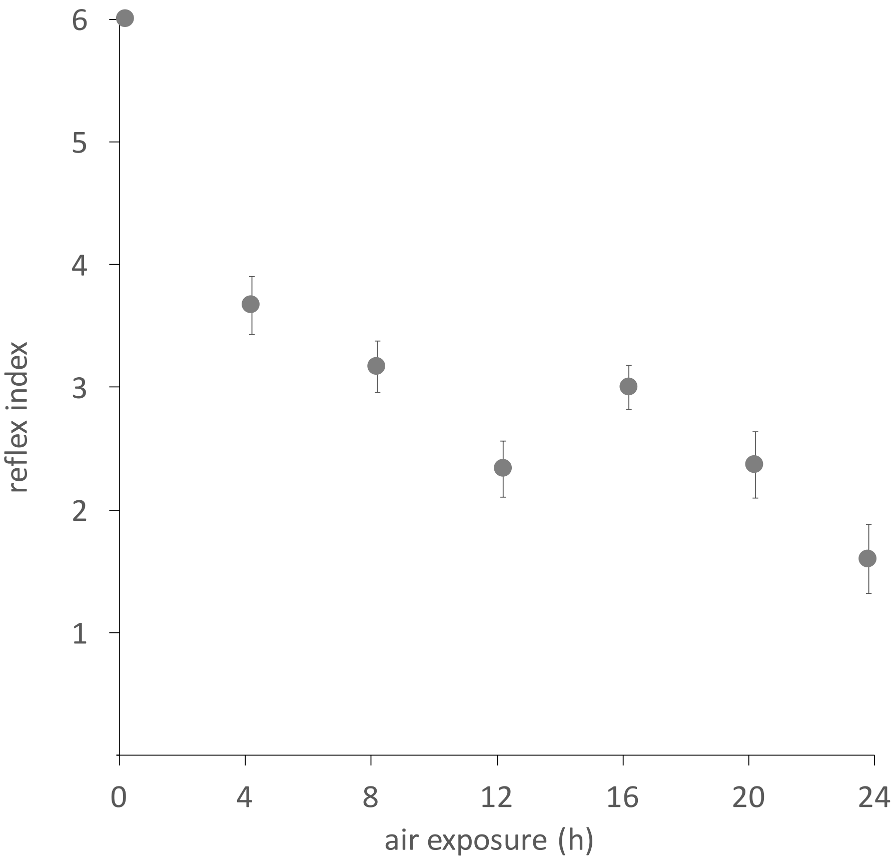
mouth, 17
6
n=36


reflex index
air exposure (h)
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8
12
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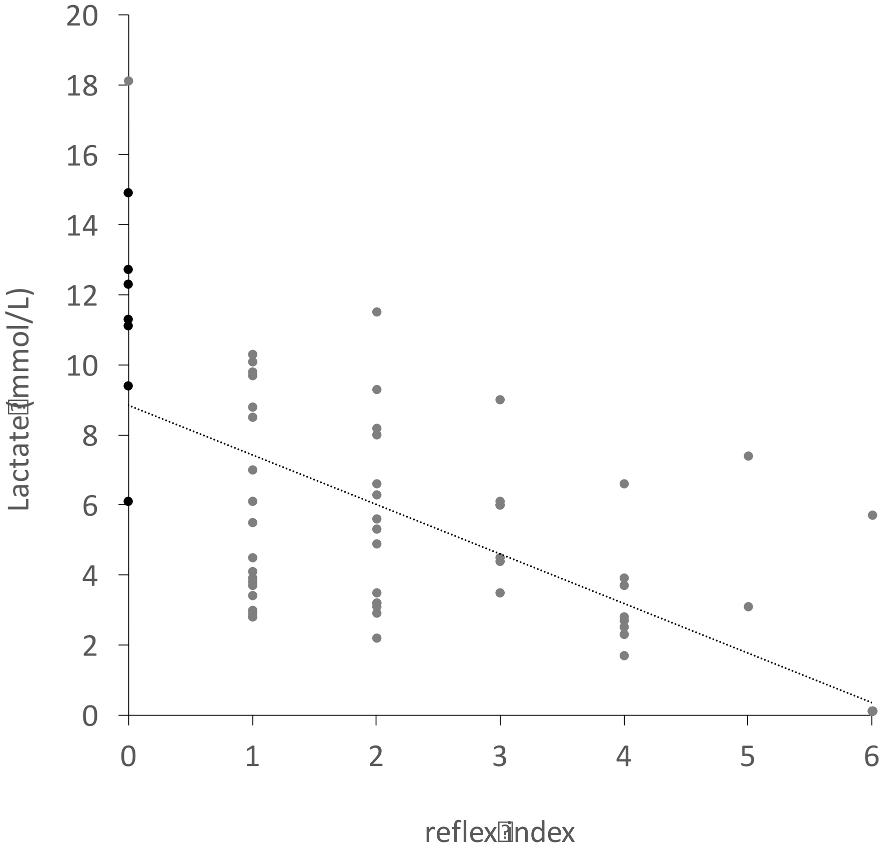
20
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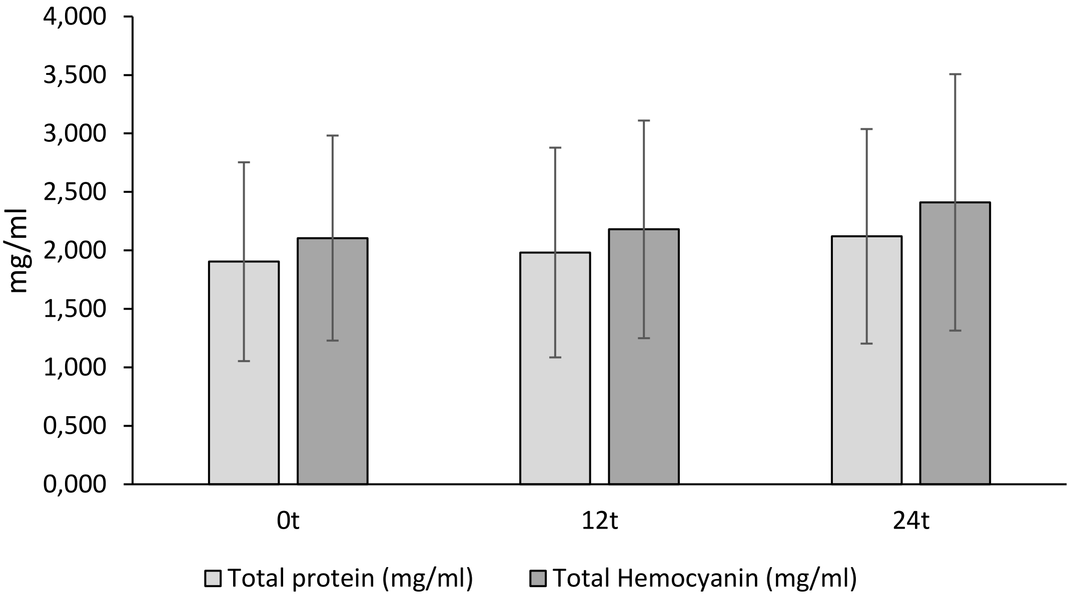
reflex index


4,000
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mg/ml
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Total protein (mg/ml)
Total Hemocyanin (mg/ml)